Male Germ Line Specific Histones of Sea Urchins and Sea Stars

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ABSTRACT

Sea urchin sperm nuclei contain two classes of histone molecules specific to the male germ line: Sp H1 and Sp H2B. Sea stars have only Sp H1. These molecules contain repeated tetrapeptide motifs of serine–proline adjacent to two basic amino acids in domains which are absent from somatic histones. The tetrapeptides are always phosphorylated except in mature sperm nuclei, where they are correlated with unusual physical properties of the chromatin including high packing densities and long nucleosomal repeat lengths. The occurrence of these molecules in the echinoderms, their modulations during spermatogenesis and following fertilization, their modes of interaction with DNA, and speculations concerning their functions are discussed.

RÉSUMÉ

Les histones spécifiques de la lignée germinale mâle des Oursins et Étoiles de Mer

Les noyaux des spermatozoïdes des Oursins contiennent deux classes de molécules d'histones spécifiques de la lignée germinale mâle: Sp H1 et Sp H2B. Les Étoiles de mer ont seulement Sp H1. Ces molécules contiennent des motifs répétés de tétrapeptides constitués de sérine-proline adjacentes à deux acides aminés basiques, dans des domaines qui sont absents dans les histones somatiques. Les tétrapeptides sont toujours phosphorylés sauf dans les noyaux des spermatozoïdes mûrs, où ils sont corrélés avec des propriétés physiques inhabituelles de la chromatine telles que de hautes densités et de grandes longueurs de répétition nucléosomiques. La discussion porte sur la présence de ces molécules dans les Échinodermes, leur modulation pendant la spermatogenèse et après la fécondation, leurs modes d'interaction avec l'ADN et des hypothèses sur leurs fonctions.

The minimal eukaryotic chromosome consists of DNA and its associated basic proteins, the histones. A histone octamer, in association with 146 bp of DNA, makes up the core nucleosome. The core contains two molecules each of the H2A, H2B, H3 and H4 histones. The remaining histone, H1, is almost always present and is associated with an additional ~20 bp of DNA to form two DNA loops about the protein core. H1 is also associated with a variable amount of linker DNA between adjacent cores. The variable linker gives rise to chromatin of different average nucleosomal repeat lengths.

The chromatin fiber may be folded into a solenoid of ~30 nm diameter or into other fiber structures. Several higher orders of packing result in the formation of highly condensed mitotic chromosomes or even more highly condensed sperm chromatin. Knowledge of these latter steps of chromatin packaging is extremely limited.

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Histones are present in virtually all cell types of all eukaryotes. The histones are distinguished by a high degree of evolutionary conservation of amino acid sequence. For example, H4 of calf and pea differ by two conservative amino acid substitutions out of 102. H3 and H4 are the most conserved, H2A and H2B less so, and H1 the most divergent. This high degree of conservation implies that histone structure is critical for proper assembly of nucleosomes and interaction with trans–acting factors affecting gene expression.

Not all nuclei contain histones. Among the earliest characterized chromatin proteins were the protamines by MIESCHER in 1897 [31]. The protamines are more basic than the histones and are not simply related to them in sequence. Their evolutionary relationship to the histones has been discussed [82, 25, see AUSIO in this volume].

MIESCHER's source of tissue was mature salmon sperm. It is now clear that sperm nuclear proteins in general are free from the evolutionary constraints operating on somatic cell histones, and the variety of non–histone structural proteins now known to be associated with sperm DNA seems at least as great as the variety of shapes that sperm display (for reviews see [25, 42]). The reason that these constraints do not apply may be that, once spermiogenesis is well underway, neither transcription, replication, nuclear transport nor mitosis have any longer to be performed. Instead, the main task is packaging of sperm DNA into a very condensed mass to be transported to the egg. It follows that in the most extreme cases of substitution of histones by other nuclear structural proteins during spermatogenesis, the transitions should take place late in spermiogenesis, after normal nuclear function ceases.

Unlike salmonids or mammals, the sea urchins and sea stars retain histones in their mature sperm cells and there are no traces of protamines or protamine–like molecules. In the urchins, however, two of the five histones, Sp H1 and Sp H2B, differ from their somatic counterparts in ways discussed below. Similar Sp H1s (but not H2Bs) are found in sea star sperm. Sp histones first appear in sea urchin spermatogenesis in the spermatogonia/spermatocytes where they completely replace somatic histones of their classes. Thus unlike protamines, the non–conserved, testis–specific echinoderm histones must function in typical nuclear processes.

In mature sperm, an additional role of Sp histones appears to be to confer unusual physical properties upon the chromatin, in particular a very large amount of linker DNA which, for the sea urchin, gives rise to the longest nucleosomal repeat lengths known, and also a high degree of chromatin compaction. How this is accomplished and speculations on the structure and function of these proteins is the subject of this article.

RESULTS AND OBSERVATIONS

Occurrence of Sp histones among echinoderms

Sp histones have been well–documented for sea urchins, established for a few sea stars and may be lacking in sea cucumbers. Ophiouroids and crinoids have to my knowledge not been investigated.

The time of radiation of the echinoderm classes including the echinoids, asteroids and holothuroids may have been about 500–600 million years ago [32, 39, 55, 63, 64]. Resolution of class relationships between echinoids, asteroids and holothuroids is not yet clear owing to difficulties in resolving estimates based on different methods and a poor fossil record for the time of their divergence [64].

All extant echinoids stem from a member of the order Cidaroida, *Myocidaris* [63]. The cidaroids or "primitive sea urchins" diverged from the euechinoids or "modern" urchins about 200 million years ago. Sp histones have been found by sequence or electrophoretic analysis in all species of sea urchin so far examined including the cidaroid *Eucidaris tribuloides* [81]. *Eucidaris* Sp H1 is about 9 amino acids shorter than that of the euechinoid *Strongylocentrotus purpuratus* H1 owing to a shorter C–terminal region. Their Sp H2Bs are of similar same size (see Table 1).

TABLE 1. — Relative sizes of Sp H1 variants. Sizes given in number of amino acid residues.

Species	Known Size [71]	Calculated Size [81]
Sea Urchin		
Parechinus angulosus	248	
Strongylocentrotus purpuratus		248 ±1.4
Eucidaris tribuloides		239 ± 0.8
Lytechinus pictus		248 ± 1.6
Lytechinus variegatus		238 ± 2.0
Arbacia punctulata		243 ± 2.8
Sea Star		
Patiria miniata (1)		251 ± 4.0
Patiria miniata (2)		260 ± 4.5

Sea star sperm (*Aphelasterias japonica, Patiria miniata, Asterias vulgaris*) lack large Sp H2B subtypes, but have large Sp H1 molecules only one of which has been sequenced [16, 30, 81, 86]. Sea cucumber (*Holothuria tubulosa*) sperm chromatin possesses somatic type histones, a sperm specific H1, and a 78–amino acid molecule resembling the C–terminal region of somatic H1s called ϕ_0 [3, 4]. The sequence of the sperm–specific H1 is not known, and therefore its relationship to Sp H1s is uncertain.

Sp histones in vivo

That sperm–specific histones of the H1 and H2B classes were present in mature sea urchin sperm was recognized with the advent of gel electrophoretic analysis [11, 37, 38, 72]. The single Sp H1 and the two or three variants of Sp H2B appeared larger than their somatic counterparts. A decade later, the first amino acid sequences of Sp histones were determined for *Parechinus angulosus* [68–71]. These demonstrated that the larger size was due mainly to N–terminal extensions on both Sp H1 and Sp H2B (and a smaller C–terminal extension on Sp H1). The N–terminal regions are rich in serine, suggesting the potential of multiple phosphorylation sites *in vivo*. In addition, Sp H1 also had a long alanine–lysine–rich region preceding the C–terminal domain (Fig 1). As discussed below, Sp histones at first approximation can be considered chimeric, a relatively conserved histone core altered by additions at the termini.

Subsequently it was found that of the five histone classes, only the Sp histones were modified in male pronuclei immediately after fertilization [17, 44, 48, 49]. These modifications were at least in part due to phosphorylation events which were mapped to serines on the N–terminal regions of Sp H2B and the N– and C–terminal regions of Sp H1 [17]. Thus sperm–specific histones are specifically phosphorylated following fertilization. Subsequently they are either displaced or diluted by histones from the egg during the first few cell divisions of the embryo [49, 59].

The origin of the Sp histones during sea urchin spermatogenesis was also investigated. Expression of the Sp H2B gene occurs in the earliest cells of the male germ lineage [51, 81]. This pattern of expression is different from that of a gene product utilized exclusively by the mature sea urchin sperm such as bindin, a major acrosomal constituent, whose mRNA is detected late in spermatogenesis [33]. This also contrasts with the expression of protamine genes in fish and

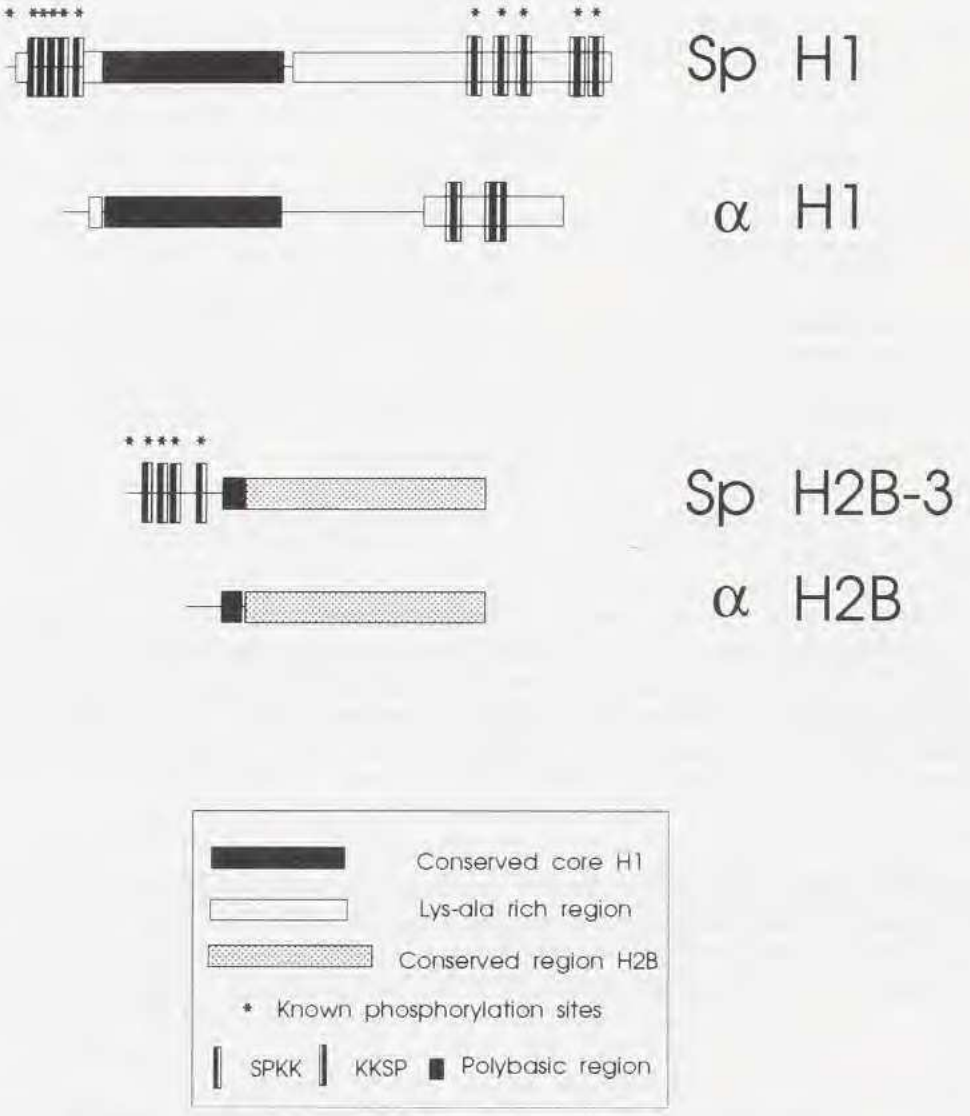

FIG. 1. — Diagram of regions in sea urchin histone variants: Comparison of Sp and somatic histones.
Derived from sequences in the Protein Identification Resource database (accession numbers: Sp H1 A02586, Sp H2B–3 A02620) and in the GenBank/EMBL database (accession numbers: embryo somatic histones H1 α, J01171, H2B α V01144).

protamines and transition protein genes in mammals which are expressed post–meiotically in spermatids [34, 46]. The cells showing expression of Sp H2B are the spermatogonia and pre–replicative spermatocytes, cells undergoing DNA synthesis. Indeed, the structure of the Sp H2B gene suggests a cell–cycle regulated gene typical of somatic histones [51].

Protamine mRNAs are long–lived and their expression is translationally controlled, the proteins appearing several developmental stages after their messages are transcribed [34, 46]. However, during sea urchin spermatogenesis, the Sp histones appear in the earliest cells of the male germ line (spermatogonia/spermatocytes) suggesting they are under transcriptional control [50]. From their first appearance until the transition from late spermatid to spermatozoon, Sp histones are phosphorylated [50]. The major phosphorylation sites in *S. purpuratus* gonadal

histones were mapped to serines in the N–terminal regions of the Sp H1 and Sp H2B [50]. All sites of phosphorylation were mapped in more detail using spermatid chromatin of *E. esculentus* [23] and shown to occur on consensus amino acid sequences SPKK (see below). As shown in Fig. 1, Sp H1 has six of these sites on serines of the N–terminal region and three sites on serines of the C–terminal region, only one of which was not in an authentic SPKK site (at SPQK). In Sp H2B, the four sites were located in the N–terminal region, one of which is at the related sequence SPSK [23].

From the parallel behavior of Sp H1 and Sp H2B phosphorylation during spermatogenesis and following fertilization, it was proposed that lack of phosphate in these histones was correlated with extreme compaction and that phosphorylation permitted the molecules to function by decreasing the affinity of the histone arms for DNA allowing the more conserved regions to dominate function [17, 43, 50]. It was suggested that the unphosphorylated arms serve to cross–link the chromatin into a compact form and stabilize it [17, 36, 82] and that this function is only exerted in the mature sperm.

The identity of the kinase responsible for SPKK phosphorylation is uncertain. Clearly it must act rapidly following fertilization. However, no reports of an endogenous sperm kinase with SPKK specificity have appeared. Eggs contain kinases which will phosphorylate the SPKK regions [14, 53, 54, 77]. SPKK sites themselves are related to cdc2 kinase sites, and are almost always present in multiple copies in C–terminal regions of somatic H1s, suggesting that they may be modified by mitotic kinases. On the other hand, inhibition of male pronuclear decondensation by amounts of the drug 6–DMAP which are sufficient to inhibit cdc2 kinase during mitosis in the same cells has little or no effect on the phosphorylation of Sp histones after fertilization [52], suggesting that the enzyme is different from cdc2, or cdc2 is differentially regulated immediately after fertilization (during G1 of the sea urchin cell cycle). Kinases of the male germ line responsible for Sp histone phosphorylation during spermatogenesis or phosphatases responsible for dephosphorylation in late spermatids are as yet uncharacterized.

Whether phosphorylation is required for removal of Sp histones from male pronuclear chromatin is not known. A protease which cleaves Sp H1 has been isolated from sea urchin eggs and suggested to be involved is such removal [76]. Its specificity for SPKK sequences is not unequivocal however, and in addition there is no evidence for proteolytic degradation of Sp H1 taking place on chromatin *in vivo*.

Primary and secondary structure of Sp histones

Sea urchin Sp H1 can be divided into four domains (Fig. 1), a conserved globular domain characteristic of all H1 molecules (amino acids 40–114), a long region rich in alanine and lysine (120–185) , and the N– and C–terminal regions characterized by multiple tetrapeptides of serine–proline adjacent to two basic amino acids (lysine and/or arginine) [43], a motif which has become known as SPKK [73].

The Sp H2B molecules (present as two or three variants in each species) can be regarded as having a well conserved C–terminal region, a 40 amino acid N–terminal region containing, in Sp H2B–1, several SPKK repeats in the first ~20 residues and a glycine–rich stretch in the next 20 amino acids followed by a conserved polybasic region rich in arginine [43, 82].

Thus the Sp H1 and Sp H2B molecules may be considered chimeric. The N–terminal regions of both and the C–terminal region of Sp H1 are unique domains related in sequence to one another which are "added" to a more conserved core molecule. The long lysine–arginine motif of Sp H1 is present in other H1s where it is shorter, may contain proline and is punctuated by SPKK motifs. Its length and lack of proline, though, make the long lysine–arginine region a unique feature of Sp H1.

TABLE 2. — Repeating tetrapeptides in Sp H1 and Sp H2b.
$X_7 = X_1X_2$SPQKR where X_1 = P or A; X_2 = G or A.
"SPKK" sequences in bold. Sea star sequence from [30]. For original references to sea urchin sequences, see [45].

Histone	Species	N–Terminal sequence							
Sp H1	*S. purpuratus*	(X7)AA	**SPRR**	**SPKK**	**SPRK**	**SPKK**		**SPRK**	RS
Sp H1	*P. angulosus*	(X7)AA	**SPRK**	**SPRK**	**SPKK**	**SPRK**	ASA	**SPRR**	KA
Sp H1	*E. crassa*	(X7)AA	**SPRK**	**SPRK**	**SPRK**	**SPKK**	K??	**SPRK**	R?
Sp H1	*S. granulosus*	(X7)AA	**SPRK**	**SPKR** G	**SPKK**	**SP??**			
Sp H1	*S. nudus*	(X7)AA	**SPRR**	**SPKK**	**SPRR**	**SPKK**	KSS	**SPRR**	K
Sp H1	*P. miliaris*	(X7)AA	**SPRK**	**SPRK**	**SPKK**	**SPRK**	ASA	**SPRX**	K
Sp H1	*E. esculentus*	(X7)AA	**SPRK**	**SPRK**	**SPKK**	**SPRK**	ASA	**SPRR**	
Sp H1	*A. vulgaris* (Sea star)	AG	**SPRK** P	**SPKK**					
Sp H2B (1)	*S. purpuratus*	PSQRSPT	**KRSP** T	**KRSP** Q					
Sp H2B (1)	*P. miliaris*	PRSKSPT	**KRSP** T	**KRSP** Q					
Sp H2B (1)	*P. angulosus*	PRSKSPT	**KRSP** T	**KRSP** T	**KRSP** Q				
Sp H2B (1)	*E. esculentus*	PSQKSPT	**KRSP** T	**KRSP** Q					
Sp H2B (2)	*S. purpuratus*	PRSPSKT	**SPRK** G	**SPRR** G	**SPSRK** A	**SPKR** G			
Sp H2B (2)	*P. miliaris*	PKSPSKS	**SPRK** G	**SPRK** G	**SPRK** G	**SPRK** G			
Sp H2B (2)	*L. pictus*	PRSPSKS	**SPRK** G	**SPRK** G	**SPRK** G	**SPRK** G			
Sp H2B (2)	*P. angulosus*	PRSPAKT	**SPRK** G	**SPRK** G	**SPSRK** A	**SPKR** G			
Sp H2B (2)	*E. esculentus*	PKSPSKS	**SPRK** G	**SPRK** A	**SPKR** G				
Sp H2B (2)	*E. crassa*	PKSPSKG	**SPRK** G	**SPRK** G	**SPTRR** G				
Sp H2B (3)	*P. angulosus*	PRSPAKT	**SPRK** G	**SPRK** G	**SPRK** G	**SPSRK** A	**SPKR** G		
Sp H2B (3)	*E. esculentus*	PRSPSKS	**SPKK** G	**SPRK** A	**SPKR** GGK				

Few sequences are available for sea star sperm histones. The complete sequence of H2B from *Asterias rubens* sperm indicates a single variant lacking in SPKK repeats [29] consistent with electrophoretic data indicating lack of sea star Sp H2Bs. Sequencing of the N–terminal region of *Asterias vulgaris* Sp H1 however shows at least two SPKK motifs are present [30] separated by a proline (Table 2). Since the partial sequence includes only the first 11 residues, additional SPKKs may be present.

The repeating amino acid sequences of Sp histones were originally described as tetrapeptides for Sp H1 and pentapeptides for Sp H2Bs [68–71]. The two basic amino acids may lie to either side of the serine–proline motif in different variants. Therefore the tetrapeptide serine–proline adjacent to two basic amino acids is the minimal common pattern which incorporates the repeated sequences present in Sp H1 and all of the Sp H2B's known (Table 2) [17, 43]. This grouping emphasizes the placement of a phosphorylatable serine near two basic amino acids. SPKK, considered a subset of SPXX, is a unit of predicted secondary structure [43] in which serine may interact with amino acids at positions 3 and 4 [73]. The nature of the third and fourth residues is not important as their interactions do not involve their side chains. The SPXX is not always coincident with SPKK in Sp histones (Table 2). The N–terminal domains containing multiple SPKK's in all Sp histones are predicted as β–turn structures [17, 36, 43, 73] or extended kinked helices [70].

In an early comparison of β–turn structures in a variety of proteins, 24 of 30 phosphorylated residues from 14 different proteins were found in β–turn regions [62]. As pointed out above this is the case *in vivo* for SPKK regions of Sp histones which are both phosphorylated and predicted β–turns. The four residues forming β–turns are often found at the surface of globular proteins and greatly affect the conformation and specificity of antibody–combining sites and enzyme active sites. They can be considered to represent cassettes which can be inserted by evolution or genetic engineering to alter the geometry of proteins [24]. SPKK "cassettes" are found in multiple copies in a variety of nucleic acid binding proteins [47].

The tetrapeptide containing region at the N–terminus of Sp H2Bs is not its only region predicted to have β–turn structure. The glycine–rich stretch between the SPKK repeats and the polybasic region of Sp H2Bs is also predicted to have β–turn structure which therefore dominates the first 40 residues of Sp H2B–1[43].

SPKK tetrapeptides have been modelled by analogy with the nucleic acid binding drugs neotropsin and Hoechst 33258 [73] and it has been suggested that they form β–turns stabilized by hydrogen bonds between the OH and CO groups of serine and the NH amide groups of the third and fourth amino acids of the tetrapeptide. More recently, a σ–turn structure has been proposed to co–exist as a conformational isomer with the β–turn in Sp H1 [78].

Measurements in solution (see below) are consistent with β–turn structure, but ultimately the question of secondary structure must be settled by crystallization of the protein both bound to and free of DNA. The only crystal structure of an H1 histone domain solved so far, for the globular region of erythrocyte H5, is a helix–turn–helix structure likely to exist in the most conserved domain of Sp H1 as well [57].

The two tetrapeptides included in the peptide SPRKSPRK are equivalent turn structures in exchange with a more extended structure by NMR analysis in solutions of DMSO [78]. The peptide may exist in a conformation between a β–turn and a σ–turn. In the latter, the amides of the two serine residues are further away and fit binding to the minor groove of DNA less well. Model building has not solved unequivocally the problem of how the binding occurs to DNA.

The long lysine–alanine stretch in Sp H1, free of proline, is predicted [43, 80] and found 15, 21] to have a high α–helical content. It has been suggested that the long α–helical stretch accommodates the extra linker in sperm DNA [21]. The long linker has been proposed to form three equivalent superhelical turns per repeat, optimizing sperm chromatin packing efficiency in some way [22]. Alternatively, the lysine–arginine–rich region can be considered a palindrome and has been modeled as two α–helices stabilizing an extra superhelical turn [75]. This reverse loop or figure–eight solenoid model would force the linker DNA into a right–handed superhelical loop towards the centre of the solenoid structure.

Interaction of Sp histones and DNA.

Most evidence supports the idea that Sp histones bind to linker DNA and that the N–terminal regions prefer the minor groove of AT–rich regions. H1 histones are known to bind to linker DNA, but Sp H2B may behave similarly. Linker binding was originally suggested on the basis of indirect evidence [17, 86], but more recent measurements confirm the proposal. Reductive methylation of lysines after their exposure following dissociation from linker DNA is consistent with binding of Sp H2B through its N–terminal region [20]. Crosslinking of DNA to Sp H2B histone also demonstrates linker binding [7]. Sp H2B in hybrid reconstituted nucleosomes protects an additional 8 bp of linker DNA against micrococcal nuclease digestion in a more efficient way than wheat H2A, which has a similar extension but lacks SPKK motifs [28].

Binding of Sp histones to linker DNA may involve short–range or long–range, intrastrand or interstrand, interactions. Although evidence for cross–linking of chromatin fibers *in vivo* by Sp histones is lacking, many studies demonstrate the unique abilities of Sp histones to bind strongly to DNA resulting in aggregation. For example, Sp H1 is more effective at aggregating

superhelical DNA than somatic H1s such as calf thymus histone [36]. In reconstitution of SV40 DNA with core nucleosomes, at intermediate ionic strengths, sea urchin core histones form large intermolecular associations which have been attributed to the Sp H2B [35].

Current data favor a model of Sp histone binding to the minor groove of AT–rich regions of DNA. Based upon binding competition studies of the N–terminal peptide of Sp H1 and Hoechst 33258, it was proposed that Sp H1 binds to the minor groove [73], although others have found no evidence for competitive inhibition [19]. Further support for binding to AT–rich regions was obtained by hydroxyl radical footprinting [8] using a synthetic peptide of two SPKK's. For a more detailed discussion of this subject, see [9].

Binding of DNA to the synthetic octapeptide SPKKSPKK conjugated to anilino–acridine was studied using DNase 1, OH–radical footprinting and osmium tetroxide. AT–selectivity of binding supported a minor groove binding model [5] and was interpreted as SPKK recognition of defined DNA sequences. Although a single tetrapeptide could unwind and extend DNA and distort the double helix, two SPKKs seem to be minimally necessary for the AT–specific minor groove binding [13].

Conformational changes in DNA have been noted upon binding of the octapeptide SPKKSPKK. This peptide without the acridine binds to calf thymus DNA or various polynucleotides. As assessed by circular dichroism, only binding to poly (dA–dT)•poly(dA–dT) induces major changes in the spectrum consistent with a φ–type condensation pattern [6]. Other peptides containing TPKK alter DNA condensation giving φ–type CD spectrum and it was concluded that the H1β–turn, although it becomes more rigid, remains a β–turn upon DNA binding [12].

What role, if any, selectivity for AT–rich regions plays in sperm chromatin structure is not clear. The stoichiometry of histone–DNA binding *in vivo* implies that Sp histones bind to all the nuclear DNA and it would be surprising if binding is not essentially uniform.

Effect of Sp histone phosphorylation on DNA binding.

Phosphorylation of the serine of the SPKK repeat should have two important consequences for its DNA binding. First the resultant addition of two negative charges per repeat should effectively neutralize the entire structure thus drastically lowering its DNA affinity. Second, by disrupting the serine hydroxyl group, the phosphate should interfere with the secondary structure.

Fewer studies have been made of the interactions of phosphorylated Sp histones with DNA than with the unphosphorylated forms. But some direct measurements support the concept of weaker binding of phosphorylated N–terminal regions to DNA. Intermolecular migration of Sp H1 which involves dissociation from the DNA is facilitated by phosphorylation [23]. Phosphorylated Sp H1 binds less tightly to DNA by affinity column chromatography [23, 73]. Multiple phosphorylation in the N–terminal but not the C–terminal regions of Sp H1 drastically reduces DNA affinity [23]. Purified N–terminal domains of Sp H1 from sperm and male germ cells were compared by several criteria including DNA cellulose binding, salt induced precipitation, thermal denaturation of DNA and inhibition of HO 33258 binding which showed that binding to DNA was always weaker for the phosphorylated peptides [19]. Unphosphorylated Sp H1 induces salt–dependent folding of long oligonucleosomes and promotes DNA self–association more effectively than phosphorylated [23].

Since sea urchin sperm chromatin has the longest repeat length known, it has been suggested that Sp H1 and Sp H2B arms might bind to and neutralize linker DNA [17, 18, 86] as discussed above. The correlation of long linker DNA in sperm chromatin with dephosphorylation and shorter linker in spermatogenic or pronuclear chromatin with phosphorylation suggested that the arms of the Sp histones might bind to linker only when their phosphates were removed [17, 18, 60]. When unphosphorylated Sp histones are bound to linker, the DNA is more resistant to

micrococcal nuclease digestion than either pronuclear [60] or spermatid [18] chromatin linkers which may be bound weakly if at all by the N–terminal arms.

Sp histones and the structure of sperm chromatin

Sperm nuclei containing unphosphorylated Sp histones have several properties which may be dependent upon these special nuclear proteins. These include high chromatin compaction, the shape of the nucleus, and the exceptional amount of linker DNA of the chromatin.

Sea urchin sperm heads are generally conical; sea star and sea cucumber spherical. Thus conical sperm nuclear shape in the echinoderms is correlated with the presence of Sp H2B, occurring only in the sea urchin. The transition from spherical to conical morphology occurs in the late spermatid, when dephosphorylation of Sp histones takes place, and the sea urchin late spermatid resembles the sea star mature sperm in nuclear shape. However, it is not clear whether nuclear shape is dependent upon dephosphorylated Sp H2B.

Sea cucumber sperm chromatin is not as highly condensed as sea urchin or sea star chromatin [40, 41]. Thus the highest degrees of chromatin compaction occur in the sea urchin and sea stars which contain a Sp H1, but again the relevance of this has not been demonstrated. *In vitro*, oligonucleosomes from sea urchin sperm seem to be more compact compared to somatic chromatins by sedimentation criteria [83]. In addition, circular dichroism measurements indicate that sea urchin sperm chromatin behaves uniquely with respect to compactness as a function of ionic strength [58].

It is impossible at present to compare in detail the higher order structure of chromatin in the nuclei of echinoderm sperm because preparation artifacts and current methodology do not permit. Sea star nuclei show regular ordered structures in minimally disrupted nuclei by electron microscopy [16]. Possibly related higher order structures have been reported in sea urchins as well [87, 88]. Chromatin fibre diameters vary between species but these are apparently not correlated with repeat length [1, 65, 85].

Whatever the exact structure of chromatin in the mature sperm nuclei, certain correlations can be made with the degree of compaction and Sp histone phosphorylation. Sea urchin sperm chromatin is packed about as densely as mitotic chromosomes [17]. Phosphorylation of Sp histones occurs prior to decondensation of the chromatin following fertilization [17]. If decondensation of chromatin is blocked by the kinase inhibitor 6DMAP, Sp histones appear to be normally phosphorylated [52]. This indicates that phosphorylation is not sufficient to induce decondensation, although it might still be necessary. Likewise it suggests that, in spite of the similarity in target sequences, the Sp kinase is not the cdc2 mitotic kinase which is inhibited under the same conditions.

The nucleosomal repeat lengths of sea star and sea cucumber sperm chromatins are shorter than sea urchin but longer than typical somatic chromatin repeat lengths (Table 3) [10, 86]. Repeat lengths increase in the sea urchin at the last stages of spermatogenesis when Sp histones are dephosphorylated [18]. In contrast, repeat lengths are constant during sea cucumber spermatogenesis [10]. Sea star and sea cucumber sperm chromatins each have repeat lengths approximately 20 base pairs (bp) shorter than sea urchin [10, 86]. Analysis of sperm chromatin of crinoids and ophiuroids is unavailable.

Thus the longest nucleosomal repeat lengths are correlated with the presence of both Sp H2B and Sp H1. Given data demonstrating that both bind to linker DNA, this is not surprising. However, sea cucumber sperm nucleosomal repeat lengths are about the same as those of sea stars. We do not know enough about the chemistry of the special sperm H1 and ϕ_o in sea cucumber to be able to speculate about their relationship to long linker.

It is not obvious whether the long linker is a consequence of Sp histone binding or is controlled by some other factor. Sea urchin sperm H1 is more efficient than somatic H1s at

TABLE 3. — Average nucleosomal repeat lengths of echinoderm sperm chromatins

Group	Species	Repeat Length (bp)	Reference
Sea Urchin			
	Eucidaris tribuloides	238 ± 2	[81]
	Strongylocentrotus purpuratus	249 ± 5; 250 ± 12	[81, 27]
	Lytechinus pictus	245 ± 5	[81]
	Arbacia lixula	241	[66]
	Strongylocentrotus intermedius	237 ± 5	[86]
	Arbacia puntulata	260 ± 26	[26]
Sea Star			
	Patiria miniata	225 ± 3; 222± 5	[81, 16]
	Aphelasterias japonica	224 ± 6	[86]
Sea Cucumber			
	Holothuria tubulosa	228 ± 5	[10]
	Thyone briareus	233	[84]

organizing nucleosomes at low packing densities in reconstitution systems *in vitro* using polyglutamic acid and poly d(A–T)•poly d(A–T) [67]. Sperm core histones (containing Sp H2Bs) are likewise more efficient at being recruited [67]. In this assay, Sp H1 gives repeat lengths approximately the same as those *in vivo*. It would be of great interest to determine if phosphorylation of Sp histones shortens the nucleosomal repeat length, using *in vitro* chromatin reconstitution systems.

DISCUSSION

Phylogenetic trees for core histones have recently been constructed [79]. The H2A/H2B histones which form dimers appear to have co–evolved and are 10 times more divergent than the H3/H4 pair. Sea urchin Sp H2B was the only divergent core histone without a correspondingly divergent H2A. It was suggested that sea urchin Sp H2Bs evolved more rapidly than other sea urchin H2Bs or H2As and that they perform a function that is atypical or assemble nucleosomes in a different way free of typical constraints. Based on the discussion above, it seems likely that Sp H2B function in the unphosphorylated state is atypical, namely the packaging of sperm chromatin, but that it can assemble proper nucleosomes when in the phosphorylated state when its conserved regions dominate its function.

It has been suggested that the repeating structure of Sp H2B is related to the structure of vertebrate protamines based upon (1) the similarity of basic residue spacing between proline and hydroxyamino acids and the nature of the basic residues (arginines) when the repeat is considered a pentapeptide of the form pro–X–X–X–ser (with two of three X's basic), and (2) the similar size of protamines and the Sp H2B N–terminal extension [82]. In this view vertebrate protamine genes would have evolved from the N–terminal region of Sp H2B toward greater basicity and more efficient DNA packaging.

However, similar motifs are not unknown among invertebrates. For example, sperm–specific protein ϕ3 from the mussel *Mytilus californianus* has two SPKK motifs punctuating an otherwise largely lysine–alanine–rich sequence [2]. This molecule of 5 kD is predominantly α–helical in structure and in some ways resembles the C–terminal region of Sp H1. In what way should we imagine its evolutionary relationship to sea urchin Sp histones?

The scheme proposed by AUSIO in this volume takes a different approach to the origin of protamines. It postulates a primitive histone precursor giving rise to somatic H1s and sperm-specific basic nuclear proteins. Echinoderm Sp histones would result from an increase in arginine content of the precursor and would co–exist with histones, whereas in other organisms intermediate type protamine–like (PL) molecules would evolve and replace somatic histones. By loss of their globular cores, these would become small PL molecules as found for example in *Mytilus*. The small PLs might then evolve to protamines. This scheme would, however, have to take into account the appearance of similar SPKK domains on the N–termini of both Sp H1s and Sp H2Bs and the possibility of their assembly from scattered SPKK domains seen in somatic H1s remains.

Another problem concerns speculation on evolutionary and functional relationships merely on the basis of amino acid sequence similarities. A good example is the central globular domain of H1 histones which is highly conserved. The crystal structure of this domain of the H1 variant H5 from erythrocytes was recently solved to 2.5 Å resolution [56, 57, 61]. Its secondary structure is remarkably similar to a liver–specific transcription factor HNF–3γ in a helix–turn–helix motif. However, sequence similarity between the two proteins is limited (12% of the structurally equivalent residues). Each is a member of a large family of proteins highly conserved in sequence and function. The choice of whether these proteins have diverged from a common ancestor or converged to a common three–dimensional structure is uncertain.

The presence of very long nucleosomal repeat lengths and Sp histone molecules in sperm of echinoids and asteroids suggests that these related molecular patterns were developed by a common ancestor and conserved to the present day. From the functional standpoint, it is not obvious why such long repeats are useful, except to allow binding of the extensions of the Sp histones. That this binding may allow a special kind of compaction of chromatin in these organisms has been discussed above. Certainly in a functional sense, the arms of Sp histones might be considered to lend protamine–like qualities to the Sp histones.

The motif SPKK or its variants (threonine instead of serine, arginine instead of lysine) is always present in the C–terminal domain of somatic H1 molecules and could represent a cassette borrowed by other molecules which would function in strong but reversible nucleic acid binding. The occurrence of the multiple tetrapeptides in a variety of DNA and RNA binding proteins has been noted [47]. It has also been argued that the related SPXX motif is found often in gene regulatory proteins [74]. Yet unfortunately too few data are available for comparisons to be made among the sperm histones of echinoderms to generalize about similarities of function of evolutionary relationships.

Since sperm cells compete for successful fertilization, they can be under great selective pressure for the brief period following spawning. This may depend on events occurring before fertilization, such as local environmental conditions, or after, such as the ability of the egg to rapidly disassemble and reassemble male chromatin. It would seem likely that efficient packaging of the sperm nucleus is an advantage in marine environments for motile sperm. However, co–evolution of egg mechanisms for formation of the male pronucleus from the sperm nucleus following fertilization may limit the rate at which sperm nuclear structural proteins can evolve.

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